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Isolation and Characterization of a New Bacterium Carboxylating Phenol to Benzoic Acid under Anaerobic Conditions

T. LI,[1] J.-G. BISAILLON,[1]* R. VILLEMUR,[1] L. LÉTOURNEAU,[1] K. BERNARD,[2] F. LÉPINE,[1] AND R. BEAUDET[1]

Centre de Recherche en Microbiologie Appliquée, Institut Armand-Frappier, Université du Québec, Québec, Québec H7V 1B7,[1] and Laboratory Centre for Disease Control, Turney's Pasture, Ottawa, Ontario K1A 0L2,[2] Canada

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A consortium of spore-forming bacteria transforming phenol to benzoic acid under anaerobic conditions was treated with antibiotics to eliminate the four *Clostridium* strains which were shown to be unable to accomplish this reaction in pure culture and coculture. *Clostridium ghonii* was inhibited by chloramphenicol (10 μg/ml), whereas *Clostridium hastiforme* (strain 3) and *Clostridium glycolicum* were inhibited by clindamycin (20 μg/ml), without the transformation of phenol being affected. Electron microscopic observations of resulting liquid subcultures revealed the presence of two different bacilli: a dominant *C. hastiforme* strain (strain 2) (width, 1 μm) and an unidentified strain 6 (width, 0.6 μm) which was not detected on solid medium. Bacitracin (0.5 U/ml) changed the ratio of the strains in favor of strain 6. *C. hastiforme* 2 was eliminated from this culture by dilution. The isolated strain 6 transformed phenol to benzoic acid and 4-hydroxybenzoic acid to phenol and benzoic acid in the presence of proteose peptone. Both of these activities are inducible. This strain is a gram-variable, flagellated rod with a doubling time of 10 to 11 h in the presence of phenol. It has a cellular fatty acid composition like that of *C. hastiforme*. However, strain 6 does not hydrolyze gelatin or produce indole. The 16S rRNA sequence of strain 6 was found to be most similar to that of some *Clostridium* species, with homology ranging from 80 to 86%. The evolutionary relationships of strain 6 to different groups of *Clostridium* and *Clostridium*-related species revealed that it does not emerge from any of these groups. Strain 6 most likely belongs to a new species closely related to *Clostridium* species.

The transformation of phenol to benzoic acid by methanogenic consortia has been the subject of many studies. However, this transformation is carried out by bacterial consortia which have been only partially characterized. From the results of feeding experiments and kinetic studies, Knoll and Winter (16) suggested that a long, nonmotile, gram-negative rod was responsible for the carboxylation of phenol to benzoic acid by the methanogenic consortium in their study. Sharak-Genthner et al. (23) found five gram-negative cell types of different shapes in their phenol-carboxylating methanogenic consortium. Zhang and Wiegel (28) have shown that their phenol-carboxylating consortium grown in the presence of yeast extract was composed of two types of rods and an organism resembling *Methanospirillum hungatei*.

Zhang et al. (27, 29) have isolated from their consortium *Clostridium hydroxybenzoicum*, which decarboxylated 4-hydroxybenzoic acid to phenol but could not further metabolize phenol. However, with resting cell suspensions and cell extracts of this strain, Zhang and Wiegel observed reversible conversion of 4-hydroxybenzoic acid and phenol (30). This is the only microorganism isolated from a methanogenic consortium that has been shown to be able to carboxylate phenol. Recently, Létourneau et al. (17) have shown the involvement of spore-forming bacteria in the carboxylation of phenol in their methanogenic consortium. However, none of the strains isolated was able to carboxylate phenol in pure culture or coculture, nor could the strains decarboxylate 4-hydroxybenzoic acid.

The difficulty in working with a methanogenic consortium arises from the fact that these bacteria are part of interdependent trophic groups, which makes the isolation of one particular strain problematic. Under denitrifying conditions, pseudomonads responsible for the carboxylation of phenol were more readily isolated (24). Also, *Desulfobacterium phenolicum* was shown to metabolize phenol and 4-hydroxybenzoate under sulfate-reducing conditions (1).

The present study focused on the isolation of the carboxylating microorganism in subcultures of the heated anaerobic consortium of Létourneau et al. (17). This was performed by using various antibiotics to eliminate unnecessary strains. Different microbiological and biochemical tests were done together with 16S rRNA sequence analysis to characterize this strain.

MATERIALS AND METHODS

Microorganisms. The anaerobic consortium of Létourneau et al. (17) heated at 80°C for 15 min was used in this study. It was maintained by serial transfers in the minimal medium of Boyd et al. (6) supplemented with 1.6 to 2.1 mM phenol (Baker Chemical Co., Phillipsburg, N.J.) and 0.05% (wt/vol) proteose peptone no. 3 (Difco Laboratories, Detroit, Mich.), using a 12.5% (vol/vol) inoculum as described previously (5). The four *Clostridium* strains (*Clostridium ghonii* 1, *Clostridium hastiforme* 2 and 3, and *Clostridium glycolicum* 5) isolated by Létourneau et al. (17) from the anaerobic consortium were used and maintained by successive transfers on Columbia blood agar. Strain 6, which was isolated in the present study, was maintained in Boyd's minimal liquid medium supplemented with phenol and 0.5% (wt/vol) proteose peptone. In all experiments, duplicate cultures were incubated at 37°C.

Antibiograms. Antibiograms were done with the four *Clostridium* strains isolated by Létourneau et al. (17) from the heated consortium. These strains were inoculated on Columbia blood agar to obtain confluent growth. Before incubation, a series of discs (Becton Dickinson Microbiology System, Mississauga, Canada) containing different antibiotics were placed on the surface of the solid media. These were incubated in an anaerobic jar for 48 h before the sensitivities of the strains were determined.

Isolation of the carboxylating strain. On the basis of the results of the antibiograms, different concentrations of chloramphenicol (Sigma Chemical Co.,

* Corresponding author. Phone: (514) 687-5010. Fax: (514) 686-5501. Electronic mail address: Jean-Guy_Bisaillon@IAF.UQUEBEC.CA.

Mississauga, Canada) were tested in liquid cultures to eliminate unnecessary strains from the consortium. Control cultures without antibiotics were used. Counts (CFU per milliliter) on Columbia blood agar following 10-fold serial dilutions of the cultures were done after 72 h of incubation to determine the activity of the antibiotics on the strains. Clindamycin phosphate (Sigma) was also used because some *Clostridium* strains have been reported to be resistant to this antibiotic (8). It was added at different concentrations (up to 100 μg/ml) to subcultures of the culture treated with 10 μg of chloramphenicol per ml. The resulting subcultures of the culture treated with 20 μg of clindamycin per ml were then incubated with different concentrations of bacitracin.

Solid (1% [wt/vol] agar) and semisolid (0.4% agar) Boyd's media were supplemented with phenol (2 mM) and proteose peptone (0.5% [wt/vol]), and cysteine (0.5 g/l) had been substituted for Na_2S. A 10^{-6} dilution of the culture treated with 0.5 U of bacitracin per ml was used as an inoculum for these media. The strain 6 maintenance liquid culture originated from a colony growing in the semisolid medium.

Electron microscopic observations. Electron microscopic observations were done with a model 7100 electron microscope (Hitachi, Tokyo, Japan). The microorganisms were first fixed with 1 to 2% glutaraldehyde and then negatively stained with 2% phosphotungstate (3). In the bacitracin experiment, the proportion of each of the strains present was determined by estimating at least 20 randomly selected fields for each culture.

Activities of the isolated strain. Strain 6 from the maintenance culture was inoculated (12.5% [vol/vol]) in the liquid medium supplemented with 0.5% (wt/vol) proteose peptone and 2 mM phenol or 4-hydroxybenzoic acid. The test compounds and their metabolites were periodically analyzed. Also, 10-fold dilutions were plated on Boyd's solid medium supplemented with phenol (2 mM) and proteose peptone (0.5% [wt/vol]) to monitor the growth of strain 6 in these cultures. In some cultures with phenol, proteose peptone was omitted from the liquid medium and replaced by yeast extract (0.5% [wt/vol]) (Gibco, Grand Island, N.Y.) or a mixture of 21 amino acids (0.024% [wt/vol] each) (Sigma).

The inducibility of the carboxylating activity of strain 6 was determined by a procedure similar to the one previously described for the original consortium (5). After growth of the strain with and without phenol in Boyd's medium supplemented with proteose peptone, the cells from both cultures were centrifuged, washed, and resuspended in fresh medium containing phenol (2 mM) with and without chloramphenicol (250 μg/ml) (Sigma). A similar experiment was done with 4-hydroxybenzoic acid (Sigma) to determine the inducibility of the decarboxylating activity of strain 6.

Analytical methods. The test compounds and their metabolites in all the cultures were periodically analyzed in duplicate by gas chromatography (GC). Extracts from acidified culture fluids were concentrated, derivatized, and analyzed by GC using a 25-m HP-5 capillary column (Hewlett-Packard, Arondale, Pa.) as described previously (4). Metabolites were identified by GC-mass spectrometry with an Ion Trap 800 (Finnigan, San Jose, Calif.) mass spectrometer, using a GC column similar to the one described above (4).

Characterization of strain 6. Strain 6 was characterized with phenotypic and cellular fatty acid (CFA) composition analyses. All biochemical tests were performed as described by Holdeman et al. (13). The strain was grown in prereduced, anaerobically sterilized medium (PRAS) containing Tween (Carr-Scarborough, Decatur, Ga.), and CFAs were extracted as described by Moore et al. (18). The samples were run on a Microbial Identification System (MIS, Newark, Del.) and analyzed against the commercial data library Moore version 3.8.

Extraction of bacterial total DNA. Liquid cultures of strain 6 and of *C. hastiforme* 2 were centrifuged at 5,000 × *g* for 10 min. The pellets were washed with phosphate-buffered saline (PBS) and centrifuged. Strain 6 cells that grew on the supplemented solid medium were recovered, washed with PBS, and centrifuged. Bacterial pellets were resuspended in TEN (50 mM Tris-HCl [pH 8.0], 20 mM EDTA, 150 mM NaCl) containing lysosyme (5 mg/ml) (Boehringer Mannheim, Laval, Canada) and incubated for 30 min at 37°C. Sodium dodecyl sulfate and β-mercaptoethanol were added to final concentrations of 0.5% (wt/vol) and 1% (vol/vol), respectively. The suspensions were frozen at −70°C in dry ice-ethanol for 10 min and thawed at 65°C. This step was repeated twice. The extracts were incubated at 45°C for 2 to 3 h with proteinase K (50 μg/ml) (Boehringer Mannheim). DNA was extracted once with phenol (saturated with Tris-HCl, pH 8.0), once with phenol-chloroform-isoamyl alcohol (25:24:1), and once with chloroform-isoamyl alcohol (24:1). DNA was precipitated with ethanol and resuspended in TE (1 mM Tris-HCl [pH 8.0], 0.1 mM EDTA). DNA was treated with 1 μg of RNase A (Boehringer Mannheim) for 15 min at 37°C.

PCR amplification of the 16S rRNA. Primers (5′-AGAGTTTGATCCTGG CTCAG-3′ and 5′-AAGGAGGTGATCCAGCCGCA-3′) representing the extremities of all eubacterial 16S rRNA genes (7) were synthesized with a Gene Assembler Plus (Pharmacia, Baie d'Urfé, Canada). PCRs were carried out in a Gene ATAQ controller (Pharmacia) with 50-μl reaction mixtures containing 100 ng of total DNA, deoxynucleoside triphosphates (200 μM each), *Pfu* DNA polymerase buffer [20 mM Tris-HCl (pH 8.75), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100, 0.1 mg of bovine serum albumin per ml], 10 pmol of each primer, and 2.5 U of *Pfu* DNA polymerase (Stratagene, La Jolla, Calif.). This mixture was heated at 80°C for 2 min before the addition of the DNA sample. The PCRs were started at 94°C for 5 min and 55°C for 5 min, followed by 30 cycles of 72°C for 2 min, 94°C for 40 s, and 55°C for 1 min. This was followed by one cycle at 72°C for 10 min.

Cloning and sequencing of the 16S rRNA gene of strain 6. The PCR product for strain 6, a unique fragment of 1.5 kb, was treated with proteinase K (Boehringer Mannheim) and the large fragment of *Escherichia coli* DNA polymerase (Pharmacia) as described by Hitti and Bertino (12). This fragment was cloned in a Bluescript vector (Stratagene) at the *Sma*I site as described by Sambrook et al. (21).

Several clones containing a 1.5-kb insert were isolated, and two of them were sequenced completely. Different parts of the 1.5-kb insert were subcloned into the Bluescript plasmid vector at appropriate restriction sites, using *E. coli* MV1190 as a bacterial host. Single-stranded DNAs were generated with M13K07 helper phage (21). Both strands of the 16S sequences were sequenced by the dideoxynucleotide method (22).

*Dde*I restriction endonuclease (Pharmacia) was used as recommended by the supplier. Electrophoretic agarose gels were made in 40 mM Tris-acetate (pH 7.2)–1 mM EDTA. In some experiments, *Sau*3A1 restriction endonuclease was also used.

Phylogenetic study. The FASTA and PILEUP programs (Genetics Computer Group, Inc.) were used to find 16S rRNA sequences similar to that of strain 6. Phylogenetic analyses were carried out with different programs on PHYLIP package 3.5 (10). The 16S rRNA sequences of 15 *Clostridium* species and of *E. coli* were aligned with the 16S rRNA sequence of strain 6 by using the PILEUP program. The 1,522 unambiguously alignable nucleotide sites from the 17 sequences were used to do a pairwise comparison using the DNADIST program with the Kimura 2-parameter (15). A distance matrix representing the number of substitutions per site for each pairwise comparison was generated. The FITCH program was used to derive the best phylogenetic tree. A bootstrap of 500 replicates was also derived with the SEQBOOT program. A distance matrix for each replicate was calculated with the DNADIST program with the Kimura 2-parameter and the FITCH program and was used to generate the best tree for each replicate. The CONSENSE program was used to derive the consensus tree. The neighbor-joining method or the unweighted pair-group means analysis method to derive the best phylogenetic tree from the distance matrix and the parsimony (DNAPARS program) and maximum-likelihood (DNAML program) methods were also used.

RESULTS

Antibiograms. All the strains were resistant to streptomycin (10 μg), neomycin (5 μg), and gentamicin (10 μg) and sensitive to chloramphenicol (30 μg) and bacitracin (10 U). However, the susceptibilities to erythromycin (15 μg), ampicillin (10 μg), penicillin (10 U), and tetracycline (50 μg) varied from strain to strain (data not shown).

Isolation of the carboxylating strain. At a concentration of 10 μg of chloramphenicol per ml, most of the phenol in the liquid medium was transformed after 36 days of incubation, but at a higher concentration (100 μg/ml) no transformation was observed. In each of these cultures, *C. ghonii* was eliminated and the three other strains were not inhibited by the antibiotic.

The resulting subculture from the culture treated with chloramphenicol (10 μg/ml) transformed phenol in the presence of clindamycin (up to 100 μg/ml). However, *C. hastiforme* 3 and *C. glycolicum* were inhibited after 15 days of incubation with ≥5 μg of clindamycin per ml. Only *C. hastiforme* 2 grew in these cultures. The addition of clindamycin (250 μg/ml) slowed or inhibited phenol transformation without affecting *C. hastiforme* 2. Pure cultures of this strain did not carboxylate phenol or decarboxylate 4-hydroxybenzoic acid.

Phenol (1.9 mM) was completely transformed to benzoic acid in the liquid media inoculated with a 10^{-6} dilution of the cultures treated with clindamycin (20 μg/ml). These cultures showed only *C. hastiforme* 2 colonies when inoculated on Columbia blood agar. However, electron microscopic observations of these cultures revealed the presence of two different rod-shaped bacteria (Fig. 1A). *C. hastiforme* 2, which grows on solid medium, is the larger bacillus (1 μm wide). An unidentified bacillus (strain 6) with a width of about 0.6 μm, which was not observed on solid medium, was also present. The ratio of the strains in a 21-day-old culture favored *C. hastiforme* 2 by approximately 5 to 1.

Bacitracin at a concentration as low as 0.5 U/ml inhibited the transformation of phenol by the clindamycin (20 μg/ml) sub-

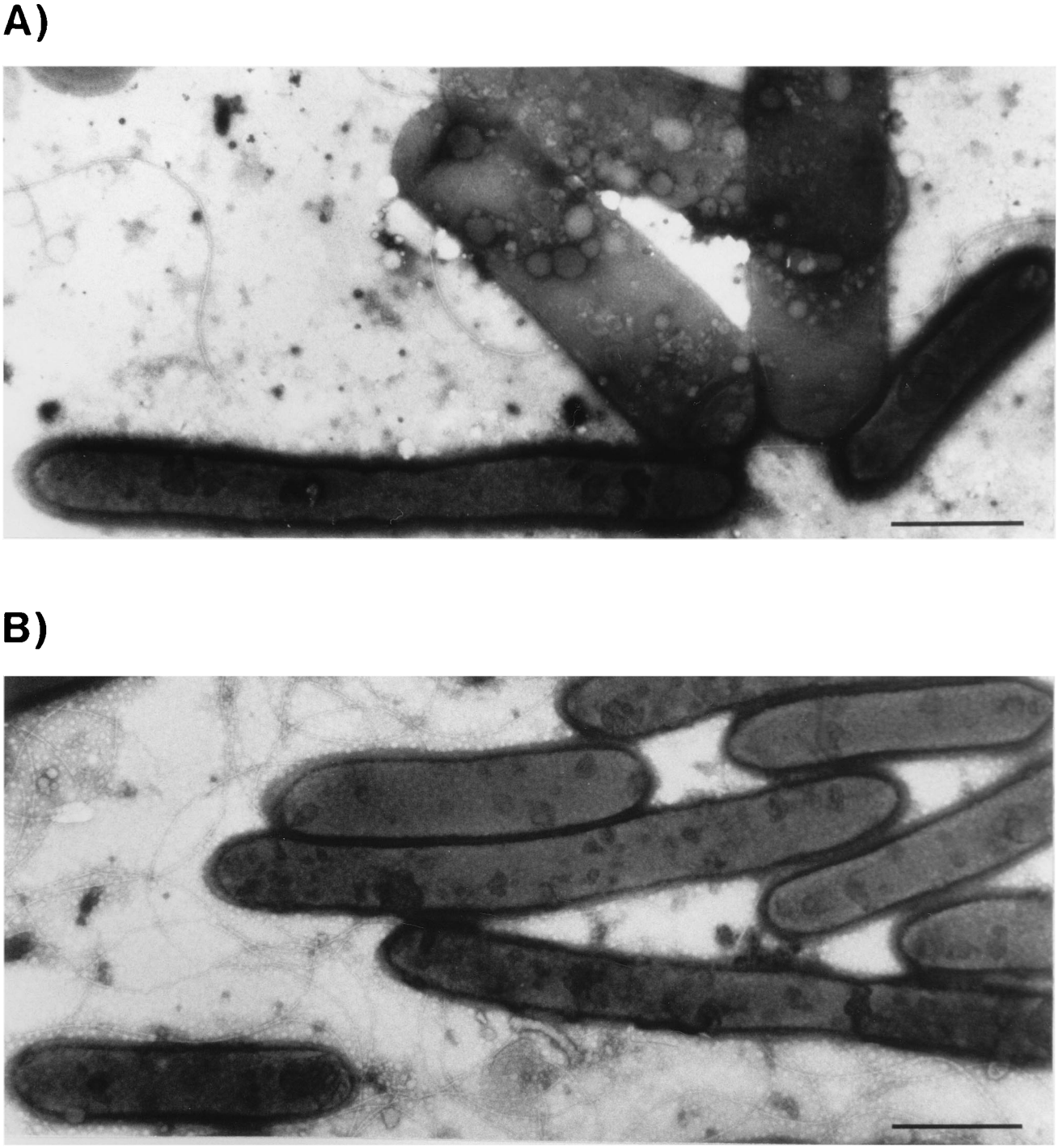

FIG. 1. Transmission electron micrographs of cells from the culture inoculated with the 10^{-6} dilution of the clindamycin-treated culture, showing *C. hastiforme* 2 and strain 6, with widths of 1 and 0.6 μm, respectively (A), and the maintenance culture, showing only strain 6 (B). Bars = 1 μm.

culture. This treatment changed the ratio between strain 6 and *C. hastiforme* 2 so that strain 6 became dominant. When this culture was diluted to 10^{-6} and inoculated in the minimal liquid medium supplemented with 0.5% (wt/vol) proteose peptone, *C. hastiforme* 2 was not present in the resulting culture, as confirmed by electron microscopy (Fig. 1B) and by the absence of growth on Columbia blood agar. Strain 6 was the only bacillus observed in these cultures, and phenol was transformed to benzoic acid.

When these cultures were inoculated on minimal solid medium supplemented with 0.5% (wt/vol) proteose peptone and phenol (2 mM), a single-colony morphology was observed after 4 days of incubation. The colonies were small, grey, and up to 0.5 mm in diameter. Strain 6 was also able to grow on Colum-

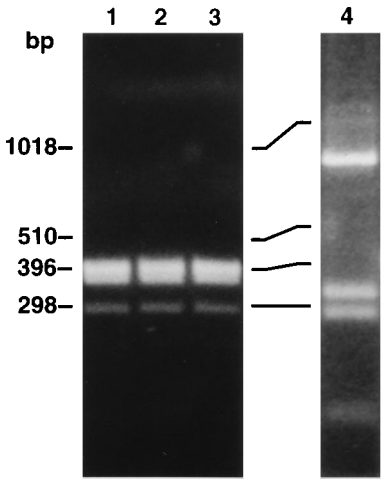

FIG. 2. Restriction endonuclease analysis of 16S ribosomal DNA. 16S ribosomal sequences from total DNAs of different bacterial strains were amplified by PCR. *Dde*I restriction digestion patterns of strain 6 (lanes 1 to 3) and of *C. hastiforme* 2 (lane 4) are shown. Lane 1, culture grown in maintenance liquid medium, with the carboxylating activity conserved; lane 2, culture grown on solid medium and having lost the carboxylating activity; lane 3, culture grown in the liquid medium after a passage on solid medium and having lost the carboxylating activity. DNAs were fractionated on a 1.8% agarose gel and visualized with ethidium bromide.

bia blood agar but only if the inoculum came from the solid medium described above and not from the liquid medium. However, this growth was slower than that on the supplemented minimal solid medium. Phenol was transformed when the entire biomass growing on the supplemented minimal solid medium was recovered and inoculated in the liquid medium but not when a single colony was used as the inoculum.

Semisolid medium was similarly inoculated. Some of the colonies growing in this medium transformed phenol when inoculated in the liquid medium. These cultures were replicated and gave rise to strain 6 maintenance cultures.

Restriction endonuclease analysis of the 16S rRNA. DNA fragments of 1.5 kb were obtained from the amplification by PCR of the 16S ribosomal sequences of strain 6 and *C. hastiforme* 2 grown in their respective maintenance cultures. The patterns observed for these fragments after digestion with restriction endonuclease *Dde*I were different (Fig. 2, lanes 1 and 4), suggesting that these strains are different. Similar DNA fragments were also obtained from strain 6 growing on the supplemented minimal solid medium and in the supplemented minimal liquid medium after inoculation from the solid medium. The patterns observed for these fragments after digestion with *Dde*I endonuclease were identical to that obtained for strain 6 grown in the maintenance culture (Fig. 2, lanes 1 to 3). Similar results were obtained after digestion with restriction endonuclease *Sau*3A1 (data not shown). This confirms that the bacterial cells obtained from the different cultures were from strain 6.

Activities of the isolated strain. In Boyd's liquid medium supplemented with 0.5% (wt/vol) proteose peptone, strain 6 grew from 10^4 to 10^7 CFU/ml in 7 days and completely transformed phenol to benzoic acid after 14 days of incubation (Fig. 3A). In this culture, the doubling time of strain 6 in the log phase of growth was 10 to 11 h. In the absence of proteose peptone, growth and carboxylating activity were not observed. Yeast extract (0.5% [wt/vol]) or a mixture of 21 amino acids (0.024% [wt/vol] each) could efficiently replace proteose peptone. Strain 6 was able to transform 4-hydroxybenzoic acid to phenol and benzoic acid after only 4 days of incubation in the presence of proteose peptone (Fig. 3B). Under these conditions, its doubling time was only 5 to 6 h.

The resuspended cells from cultures grown with 4-hydroxybenzoic acid exhibited faster transformation of 4-hydroxybenzoic acid than the cells from cultures grown without the compound (Table 1). In the presence of chloramphenicol, only the cells previously grown with 4-hydroxybenzoic acid transformed this compound. Similar results were obtained in the phenol experiment (data not shown).

Characterization of strain 6. Strain 6 is a gram-variable, motile, anaerobic bacillus. It grows on supplemented brain heart infusion agar after 2 days of incubation under anaerobic conditions but not aerobically, in CO_2, or under microaerophilic conditions. Colonies were circular, slightly convex, grey, opaque, and mottled. Strain 6 has a CFA composition like that of *C. hastiforme*, with a similarity index of 0.33 to 0.635 after growth in PRAS containing Tween. However, it can be differentiated from this species since indole is produced and gelatin

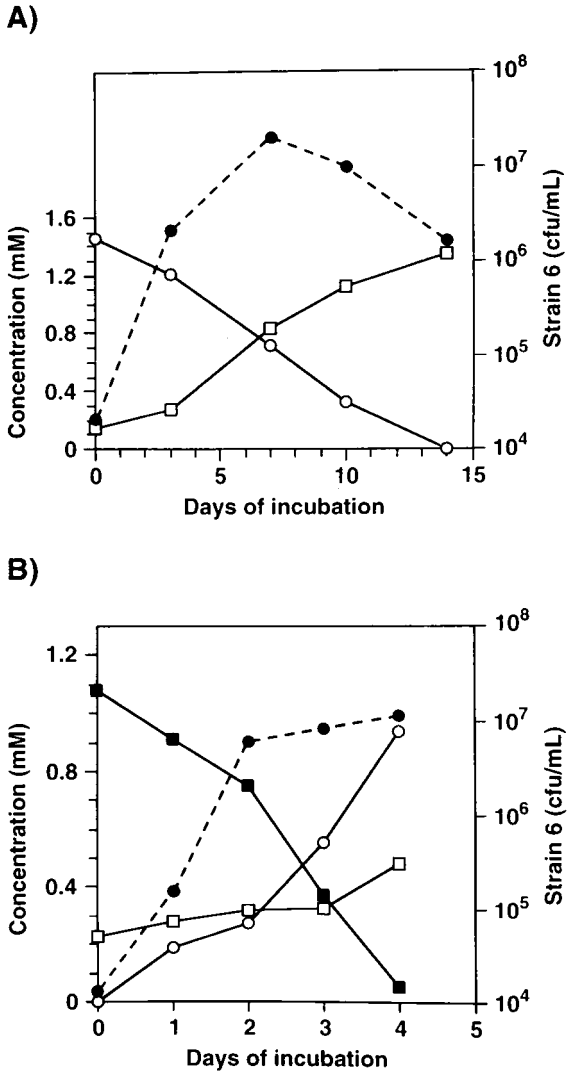

FIG. 3. Kinetics of phenol (A) and of 4-hydroxybenzoic acid (B) transformation by strain 6 in Boyd's medium supplemented with 0.5% (wt/vol) proteose peptone. ○, phenol; ■, 4-hydroxybenzoic acid; □, benzoic acid; ●, CFU per milliliter.

TABLE 1. Transformation of 4-hydroxybenzoic acid to phenol and benzoic acid by 4-hydroxybenzoic acid-induced and -noninduced cultures of strain 6 in the presence and absence of chloramphenicol

Days of incubation	Concn (mg/liter)[a]											
	4-OHB-induced culture						Noninduced culture					
	Without chloramphenicol			With chloramphenicol			Without chloramphenicol			With chloramphenicol		
	4-OHB	P	B	4-OHB	P	B	4-OHB	P	B	4-OHB	P	B
0	326	2	2	338	2	2	357	1	6	327	2	3
3	28	153	38	303	19	2	377	2	2	337	2	3
8	1	126	126	280	40	5	270	6	70	342	2	3
11	11	103	161	252	52	2	5	145	104	332	2	3
18	12	53	256	247	66	2	1	116	168	340	2	3
24	ND[b]	ND	ND	216	85	6	2	92	225	355	3	<1

[a] 4-OHB, 4-hydroxybenzoic acid; P, phenol; B, benzoic acid.
[b] ND, not determined.

is not hydrolyzed. Carbohydrates (fructose, glucose, lactose, maltose, mannitol, mannose, melibiose, raffinose, ribose, salicin, starch, sucrose, and xylose) are not fermented even when supplemented with serum, which is consistent with *C. hastiforme* as well as a number of other species of *Clostridium*. Esculin was not hydrolyzed; milk and meat were not digested; nitrate was not reduced; oxidase, catalase, lecithinase, and lipase were not produced; and hemolysis was not observed. Electron microscopic observations revealed that the bacilli were flagellated (Fig. 1B), and their aspect was consistent with gram-positive bacteria. Spores were not observed, but when grown on Wagenaar and Dack medium, the extremities of some cells were distended. The maintenance culture was not heat resistant (70°C for 10 min), but strain 6 and 2 cocultures (from the culture treated with 20 μg of clindamycin per ml) were resistant to a treatment of 80°C for 15 min.

Phylogenetic study of strain 6. The sequencing of two clones revealed that each had an insert of 1,514 nucleotides corresponding to 16S rRNA genes of strain 6. These two sequences were identical except for seven differing nucleotides clustered in two short regions (positions 64 to 68 and 88 to 93). Another clone was sequenced at this particular region and the same differences were found, suggesting that the two sequences are from different 16S rRNA genes. No identical 16S rRNA sequence was found in gene banks. However, 34 of the 50 most similar sequences were from *Clostridium* species, with identity ranging from 80 to 86%. Several progressive, pairwise alignments with the strain 6 16S rRNA sequence and sequences of single representatives of different families and genera of bacteria (19) were made with the PILEUP program. These analyses also showed that strain 6 was closely related to *Clostridium* species.

The evolutionary relationship of strain 6 to different *Clostridium* and *Clostridium*-related species was determined by 16S rRNA gene sequence analysis. The species chosen for this analysis are representative of different groups or clusters of *Clostridium* and *Clostridium*-like species based on the classification of Collins et al. (9). Pairwise comparisons of the sequences were made to determine the evolutionary distance

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 1-C. hydroxybenzoicum
 2-C. ghonii           0.186
 3-C. thermocellum     0.208 0.224
 4-C. thermiditis      0.222 0.239 0.096
 5-C. thermolacticum   0.202 0.230 0.100 0.137
 6-Strain 6            0.200 0.233 0.167 0.151 0.177
 7-C. cellulosi        0.214 0.264 0.151 0.172 0.171 0.187
 8-C. butyricum        0.203 0.228 0.178 0.200 0.220 0.193 0.219
 9-C. proteolyticum    0.202 0.209 0.182 0.197 0.215 0.201 0.196 0.102
10-C. putrificum       0.206 0.217 0.178 0.200 0.203 0.182 0.205 0.105 0.100
11-C. pfennigii        0.190 0.220 0.171 0.189 0.195 0.183 0.206 0.177 0.159 0.169
12-Th. brockii         0.219 0.220 0.193 0.212 0.192 0.224 0.215 0.236 0.233 0.240 0.212
13-C. thermoaceticum   0.189 0.200 0.170 0.196 0.173 0.210 0.197 0.220 0.213 0.217 0.193 0.129
14-C. ferridus         0.193 0.208 0.151 0.189 0.156 0.186 0.176 0.165 0.164 0.166 0.165 0.170 0.164
15-D. australicum      0.251 0.259 0.220 0.253 0.214 0.274 0.253 0.282 0.276 0.288 0.261 0.193 0.171 0.211
16-T. xylanolyticum    0.214 0.226 0.185 0.203 0.215 0.219 0.211 0.224 0.216 0.229 0.200 0.155 0.158 0.170 0.219
17-E. coli             0.314 0.308 0.266 0.298 0.270 0.282 0.288 0.287 0.288 0.296 0.302 0.281 0.280 0.271 0.328 0.301
                         1     2     3     4     5     6     7     8     9    10    11    12    13    14    15    16
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FIG. 4. Evolutionary distances between strain 6 and related *Clostridium* and *Clostridium*-like species. T, *Thermoanaerobacterium*; Th, *Thermoanaerobacter*; D, *Desulfotomaculum*.

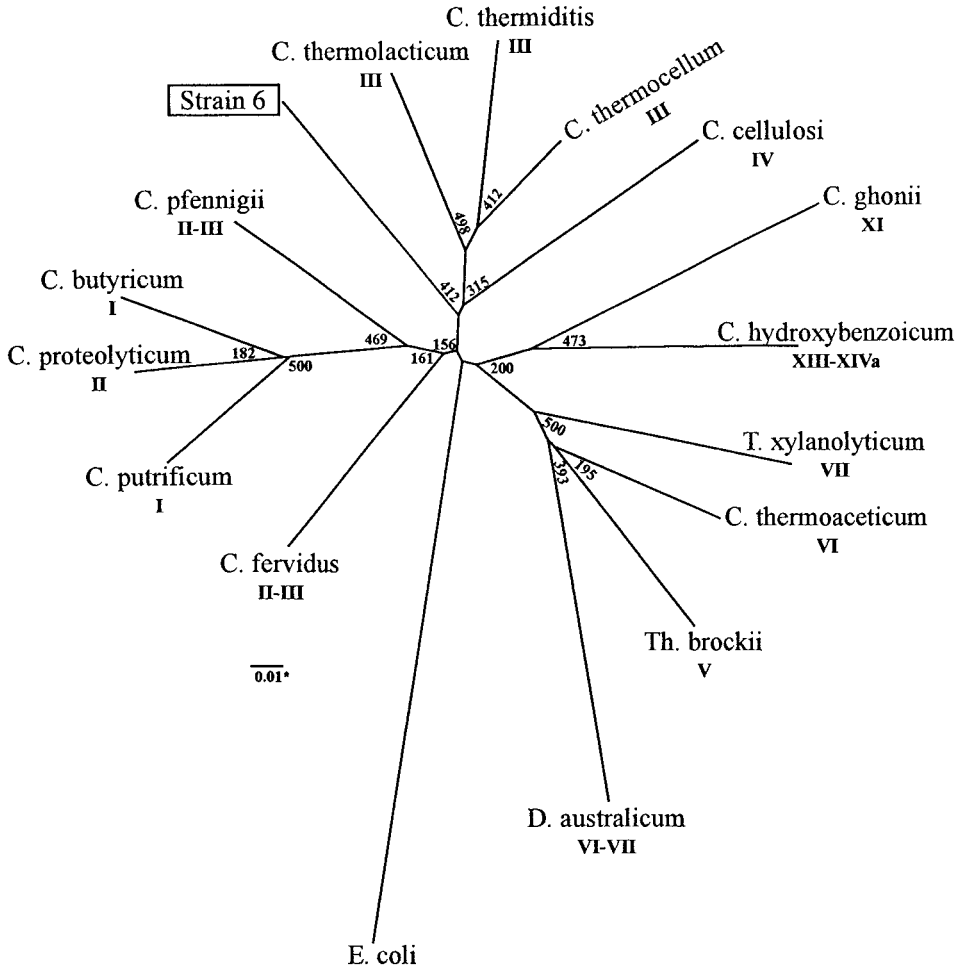

FIG. 5. Phylogenetic tree based on 16S rRNA gene sequences of strain 6 and related *Clostridium* and *Clostridium*-like species. Species whose sequences were used for comparison (and GenBank accession numbers) were as follows: *Clostridium proteoliticum* (X73448), *Clostridium thermiditis* (X71854), *Clostridium pfennigii* (X77838), *Clostridium putrificum* (X73442), *Clostridium fervidus* (L09187), *Clostridium thermolacticum* (L09176), *Clostridium butyricum* (X68176), *Clostridium thermocellum* (L09173), *Clostridium cellulosi* (L09177), *Thermoanaerobacter brockii* (L09165), *C. hydroxybenzoicum* (L11305), *C. ghonii* (X73451), *Thermoanaerobacterium xylanolyticum* (L09172), *Desulfotomaculum australicum* (M96665), *C. thermoaceticum* (M59121), and *E. coli* (J01695). Roman numerals indicate the clusters in which the *Clostridium* or *Clostridium*-like species belong. The tree was derived from the FITCH program on the PHYLIP package, using the distance in Fig. 4 with the *E. coli* 16S rRNA gene as an outgroup species. A bootstrap analysis of the same sequences was done with the SEQBOOT program with 500 replicates. The distances were calculated from each replicate with the DNADIST program using the Kimura 2-parameter option. The FITCH program derived the best tree for each replicate. The CONSENSE program was used to derived the consensus tree. The numbers at the forks indicate the number of times the group consisting of the species originating from that fork occurred among 500 trees. *, evolutionary distance (in nucleotide substitutions per site).

between each pair of sequences (Fig. 4). The best phylogenetic tree was derived from these distances (Fig. 5). This analysis showed that strain 6 does not emerge from clusters I and II, which represent the main core of the *Clostridium* genus (9). It also does not emerge from other clusters, as it branches deeply into the tree. Similar results were obtained following analysis with other phylogenetic methods (parsimony and maximum likelihood). Strain 6 is most likely a new *Clostridium*-like species that is not related to any clusters of *Clostridium* or *Clostridium*-like species.

DISCUSSION

Strain 6, a phenol-carboxylating microorganism, was isolated by its resistance to heat (17), chloramphenicol, and clindamycin and its low sensitivity to bacitracin. To our knowledge, there is only one other microorganism (*C. hydroxybenzoicum*) able to carboxylate phenol that has been isolated from a methanogenic consortium (27, 29). Our experimentation focused on liquid culture since Létourneau et al. (17) had shown that none of the strains from the heated consortium which were isolated on solid medium were able to transform phenol in pure culture or coculture.

The strategy of using antibiotics to obtain a carboxylating microorganism in pure culture was based on the fact that *Clostridium* spp. are known to vary widely in their susceptibility to some antibiotics (8). *C. ghonii*, *C. glycolicum*, and *C. hastiforme* 3 are not implicated in the carboxylation of phenol, since they were eliminated from the consortium by chloramphenicol and clindamycin without this reaction being affected. Those species are known to be susceptible to these antibiotics (8). Moreover, *C. hastiforme* 2 is not the carboxylating microorganism, since it was resistant to a high concentration of clindamycin (250 μg/ml) while phenol transformation was inhibited under these conditions, suggesting the presence of an unknown microorganism. Electron microscopic observations confirmed this hypothesis. *C. hastiforme* 2 was more susceptible

to bacitracin than strain 6, and thus it was eliminated by dilution.

Like the *C. hydroxybenzoicum* strain isolated by Zhang et al. (27, 29) and the *Clostridium thermoaceticum* ATCC 39073 strain studied by Hsu et al. (14), the strain we isolated was able to transform 4-hydroxybenzoic acid to phenol. However, our strain was the only one to further metabolize phenol produced from this reaction. *C. hydroxybenzoicum* showed some carboxylating activity only when resting cell suspensions or cell extracts were in the presence of a high concentration of phenol (10 mM) (30). A long, nonmotile, gram-negative rod presumably possessing both these carboxylation and decarboxylation activities was observed by Knoll and Winter (16) in feeding experiments with their methanogenic consortium, but it was not isolated. The nitrate-reducing *Pseudomonas* sp. described by Tschech and Fuchs (24, 25) can accomplish both reactions. Several other anaerobic bacteria, including *D. phenolicum* (1), are able to metabolize 4-hydroxybenzoic acid and phenol.

Strain 6 carboxylates phenol by cometabolism, as previously shown for the original consortium (2). Amino acids or their degradation intermediates are probably the carbon and energy sources of this strain, as suggested elsewhere (3). *C. hydroxybenzoicum* is also known as an amino acid-utilizing microorganism (27).

The data obtained for strain 6 confirm previous results presented for the original consortium on the inducibility of the carboxylating activity by phenol (5). They also add the finding that the decarboxylating activity is inducible. *C. hydroxybenzoicum* also produces an inducible enzyme that catalyzes the decarboxylation of 4-hydroxybenzoic acid (27). This enzyme was purified and shown to also catalyze the reverse reaction, namely, the carboxylation of phenol to 4-hydroxybenzoic acid (11). The purification of the phenol-carboxylating enzyme of strain 6 should reveal if there is one enzyme with both activities or if there are two different enzymes.

By its CFA composition, strain 6 was shown to be related to *C. hastiforme*, but it differs from this species in other properties (hydrolysis of gelatin and production of indole). This carboxylating strain, devoid of spores and of heat resistance, also differs from characteristics generally observed for *Clostridium* spp. However, the heat stability (80°C, 15 min) and ethanol resistance of the carboxylating microorganism present in the original consortium have been repeatedly observed, and Létourneau et al. (17) concluded that spore-forming microorganisms were involved. In addition, strain 6 and 2 cocultures survived a similar heat treatment. Such a discrepancy has previously been observed by Utkin et al. (26) for a dechlorinating activity between their enrichment culture and the purified organism. Some *Clostridium* spp. are known to sporulate rarely even if special media or conditions are used. Thus, spore demonstration is sometimes difficult. For example, fresh isolates of *Clostridium spiroforme* from humans may not form spores or survive at 70°C for 10 min (8). It is possible that some factors which are present only in the consortium culture or coculture are needed for the sporulation of strain 6.

No 16S ribosomal sequences that were identical or nearly identical to the 16S sequence of strain 6 were found in GenBank. The phylogenetic analysis revealed that the carboxylating microorganism is closely related to the genus *Clostridium*. However, this genus has been recognized to be very heterogeneous. Several *Clostridium* species have been found to be phylogenetically related to other spore-forming and non-spore-forming genera. Collins et al. (9) proposed a new classification of the *Clostridium* genus. They grouped together several *Clostridium* and *Clostridium*-like species as clusters on the basis of phenotypic criteria and phylogenetic analyses. Clusters I and II would make the core of the *Clostridium* genus, and the other clusters would form new families and genera. Strain 6 does not emerge from clusters I and II, and, under the proposed system, it would not be considered a true *Clostridium* species. Strain 6 also does not emerge from other clusters, although it may be classified in the vicinity of clusters III and IV. Species in these two clusters are polysaccharolytic *Clostridium* spp., such as *Clostridium thermocellum*, *Clostridium thermiditis*, *Clostridium cellubioparum*, *Clostridium papyrosolvens*, *Clostridium aldrichii*, *Clostridium cellulolyticum*, and *Clostridium thermolacticum* (20). However, since strain 6 was shown to be asaccharolytic, these species differ from it physiologically. Also, because strain 6 branches deeply in the phylogenetic tree, it is more likely that it is not related to any particular cluster of *Clostridium* spp.

Strain 6 is also different from the newly isolated *C. hydroxybenzoicum* of Zhang et al. (27) (Fig. 5). The isolated carboxylating strain most likely belongs to a new *Clostridium*-like species. This would explain why strain 6 could not be classified in any known species on the basis of its characteristics. In order to confirm this hypothesis, experiments to attempt to induce sporulation and heat resistance in strain 6 are under way in our laboratory.

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